Storage and regulated secretion of factor VIII in blood outgrowth endothelial cells

van den Biggelaar, M.; Bouwens, E.A.M.; Kootstra, N.A.; Hebbel, R.P.; Voorberg, J.; Mertens, K.

Published in:
Haematologica

DOI:
10.3324/haematol.13427

Citation for published version (APA):
Background
Gene therapy provides an attractive alternative for protein replacement therapy in hemophilia A patients. Recent studies have shown the potential benefit of directing factor (F)VIII gene delivery to cells that also express its natural carrier protein von Willebrand factor (VWF). In this study, we explored the feasibility of blood outgrowth endothelial cells as a cellular FVIII delivery device with particular reference to long-term production levels, intracellular storage in Weibel-Palade bodies and agonist-induced regulated secretion.

Design and Methods
Human blood outgrowth endothelial cells were isolated from peripheral blood collected from healthy donors, transduced at passage 5 using a lentiviral vector encoding human B-domain deleted FVIII-GFP and characterized by flow cytometry and confocal microscopy.

Results
Blood outgrowth endothelial cells displayed typical endothelial morphology and expressed the endothelial-specific marker VWF. Following transduction with a lentivirus encoding FVIII-GFP, 80% of transduced blood outgrowth endothelial cells expressed FVIII-GFP. Levels of FVIII-GFP positive cells declined slowly upon prolonged culturing. Transduced blood outgrowth endothelial cells expressed 1.6±1.0 pmol/1×10⁶ cells/24h FVIII. Morphological analysis demonstrated that FVIII-GFP was stored in Weibel-Palade bodies together with VWF and P-selectin. FVIII levels were only slightly increased following agonist-induced stimulation, whereas a 6- to 8-fold increase of VWF levels was observed. Subcellular fractionation revealed that 15-22% of FVIII antigen was present within the dense fraction containing Weibel-Palade bodies.

Conclusions
We conclude that blood outgrowth endothelial cells, by virtue of their ability to store a significant portion of synthesized FVIII-GFP in Weibel-Palade bodies, provide an attractive cellular on-demand delivery device for gene therapy of hemophilia A.

Key words: factor VIII, von Willebrand factor, endothelial progenitor cells, Weibel-Palade bodies, gene therapy.

Citation: van den Biggelaar M, Bouwens EAM, Kootstra NA, Hebbel RP, Voorberg J, and Mertens K. Storage and regulated secretion of factor VIII in blood outgrowth endothelial cells. Haematologica 2009; 94:670-678. doi:10.3324/haematol.13427

©2009 Ferrata Storti Foundation. This is an open-access paper.
Introduction

Hemophilia A is an X chromosome-linked bleeding disorder affecting 1-2 in 10,000 males. It results from quantitative or qualitative defects of blood coagulation factor (F)VIII. Current treatment includes protein replacement therapy with plasma-derived or recombinant FVIII concentrates. However, due to its short half-life in circulation (t½ approximately 12 h), treatment requires frequent intravenous infusions of FVIII concentrates. Gene therapy or cell-based approaches have the potential to provide a life-long, cost-effective cure for hemophilia A. A number of clinical trials for hemophilia A gene therapy have been performed. So far, limited clinical efficacy of gene delivery approaches has been observed. This is primarily caused by limitations in obtaining sufficiently high levels of FVIII in the circulation. This may be partly due to the fact that the exact cellular site of FVIII biosynthesis has not been clearly identified. Recent studies have suggested that it may be beneficial to express FVIII in cells that also express its natural carrier protein von Willebrand factor (VWF). In the circulation, VWF protects FVIII from premature clearance and proteolytic degradation by virtue of its ability to bind to it with high affinity.

VWF is expressed in megakaryocytes and vascular endothelial cells. In endothelial cells, synthesis of VWF drives the formation of elongated cigar-shaped storage organelles called Weibel-Palade bodies (WPBs) that release their content upon agonist-induced stimulation. Expression of FVIII in endothelial cells results in storage of FVIII in WPBs. Co-storage of the VWF/FVIII complex in secretory granules and subsequent release of the VWF/FVIII complex upon agonist-induced stimulation has the potential of secreting large amounts of FVIII at sites of vascular injury as well as directly increasing FVIII half-life by protecting FVIII from premature clearance and proteolytic degradation.

Endothelial (progenitor) cells have been used for cell-based therapy as well as for gene therapy. Studies in hemophilia A mice have demonstrated that transplantation of liver sinusoidal endothelial cells can correct the hemophilic phenotype. In addition, transplantation of genetically modified BOECs intravenously or implanted subcutaneously in a Matrigel scaffold results in long-term therapeutic levels of FVIII.

The above mentioned studies have demonstrated proof of principle that endothelial cells are capable of long-term synthesis and secretion of large amounts of FVIII. However, quantitative aspects of FVIII secretion and storage in endothelial cells have not yet been explored. Here, we analyzed the intracellular routing of FVIII and VWF in genetically modified BOECs using fluorescently tagged FVIII. We demonstrate that part of the synthesized FVIII-GFP is present within WPBs, providing a reservoir of FVIII that can be released following vascular perturbation.

Materials

All chemicals used were of analytical grade. Endotoxin Free Plasmid Isolation kits were from Qiagen (Hilden, Germany). Ficoll-Paque Plus was from GE Healthcare (Uppsala, Sweden). Fetal Calf Serum (FCS) was from HyClone (Logan, UT, USA). One shot Sbf chemically competent cells, Hank’s Balanced Salt Solution (HBSS), antibiotic/antimycotic, trypsin, DMEM (4.5 g/L glucose), RPMI-1640 and M199-Hepes were obtained from Invitrogen (Breda, the Netherlands). DNA modifying enzymes were from Fermentas (St Leon-Rot, Germany). Streptomycin and penicillin were from BioWhittacker (Verviers, Belgium). Endothelial Cell Medium-2 (ECM-2) was obtained from Lonza (Walkersville, MD, USA). Collagen type 1 rat tail was from BD Biosciences (Upsala, Sweden). Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, epinephrine, thrombin, isobutylmethylxanthine (IBMX) and brefeldin A (BFA) were from Sigma-Aldrich (St-Louis, MO, USA). Culture plates, cell factory (6520 cm²) and microtiterplates (Maxisorp) were from Nunc (Roskilde, Denmark).

Production of viral vectors

The lentiviral (LV) packaging system consists of three constructs encoding gag/pol (pMDL.RRE), vesicular stomatitis virus glycoprotein envelope (pCMV-VSV-G) and rev (pRSV-Rev). The self-inactivating lentiviral vector construct pLV CMV-GFP has been described before. A lentiviral self-inactivating vector encoding human B-domain deleted FVIII under control of the CAG promoter consisting of the chicken β-actin promoter, CMV enhancers and a large synthetic intron has been previously described.

FVIII-GFP with GFP replacing the B-domain in pcDNA3.1 has also been described before. The lentiviral vector in pcDNA3.1 was exchanged for fresh medium containing 100 mg/L sodium butyrate. Viral supernatant was collected after an additional 48 h incubation period, filtered through a 0.45 µm Durapore (PVDF) membrane (Millipore, Billerica, MA, USA), and concentrated by centrifugation at 50,000g for two hours. Lentiviral vectors were resuspended in HBSS and stored in aliquots at -80°C. Vector titers were determined by transduction of 1×10⁶ 293T cells with serially diluted vector followed by flow cytometry. Titers were calculated with the following formula:

\[ \text{Titers} = \frac{\text{number of cells at time of transduction}}{10^6} \times \text{percentage} \times \text{volume of viral preparation added in mL} \]
Blood outgrowth endothelial cell isolation and transduction

BOECs were isolated essentially as described from 50 ml venous blood donated by healthy volunteers. Passage five cultured BOECs (2.5×10^5) were transduced following a single exposure to pLV CMV-GFP (MOI 10) or pLV CAG-FVIII-GFP (MOI 7.5) in the presence of 5 μg/mL polybrene (Janssen Chimica, Beerse, Belgium) and centrifuged for 90 min at 300 g and 32°C. After a four hour total incubation time, medium was refreshed, and transduced cells were further cultured using standard BOEC cell culture procedures. To determine steady state production levels, conditioned medium was collected, centrifuged for 10 min at 10,000 g, supplemented with 10 mM benzamidine and stored at -20°C until use.

Flow cytometry

Percentage of GFP positive cells was determined by flow cytometry. Cells were washed twice with HBSS, lifted with 0.05% trypsin-EDTA and were resuspended in 1% (w/v) bovine serum albumin (BSA) (Albumin Fraktion V, Merck, Darmstadt, Germany) in phosphate buffered saline (PBS) supplemented with 10% (v/v) Cellfix (BD Biosciences, Uppsala, Sweden). Flow cytometry was performed using the LSR II (BD Biosciences, Uppsala, Sweden). The acquired data were analyzed with FACSDiva software (BD Biosciences, Uppsala, Sweden).

Quantification of FVIII and von Willebrand factor

FVIII antigen was quantified using an anti-light chain ELISA. Monoclonal anti-FVIII light chain antibody CLB-CAg12 was coated into a 96-well microtiterplate (5 μg/mL; 100 μL/well) in 50 mM NaHCO3 (pH 9.6) for at least 16 h at 4°C. Plates were washed with 0.1% (v/v) Tween20, PBS (pH 7.4). Samples were diluted in 2% (v/v) human serum albumin (HSA) (Cealb, Sanquin, Amsterdam, the Netherlands), 1 M NaCl, 50 mM Tris (pH 7.4) and incubated with the immobilized antibody for two hours at 37°C. Peroxidase-labeled monoclonal anti-FVIII light chain antibody CLB-CAg17 was used to detect bound FVIII light chain. Normal human pooled plasma was used as standard. FVIII activity was quantified using a chromogenic assay according to the manufacturer’s instructions (Chromogenix, Milan, Italy). Human VWF antigen was quantified using an ELISA. Monoclonal anti-VWF antibody CLB-Rag20 was coated into a 96-well microtiterplate (1 μg/mL; 100 μL/well) in 50 mM NaHCO3 (pH 9.6) for at least 16 h at 4°C. Plates were washed with 0.1% (v/v) Tween20, phosphate buffered saline (PBS) (pH 7.4). Samples were diluted in 0.1% (v/v) Tween20, 1% (v/v) bovine serum albumin (BSA) (Albumin Fraktion V, Merck, Darmstadt, Germany), PBS (pH 7.4) and incubated with the immobilized antibody for two hours at 37°C. Peroxidase-labeled polyclonal rabbit anti-human VWF antibody (DAKO, Glostrup, Denmark) was used to detect bound VWF. Normal human pooled plasma was used as standard.

Immunofluorescence microscopy

Non-transduced BOECs were grown on 1 cm-diameter gelatin-coated glass coverslips. Confluent cells were fixed with PBS/3.7% paraformaldehyde (PFA) and prepared for immunofluorescence analysis as described. Monoclonal antibody CLB-Rag20 was used to visualize VWF. Rabbit polyclonal antibody anti-human CD62-P (BD PharMingen, San Diego, CA, USA) was used to visualize P-selectin. Monoclonal antibody CLB-HEC751 was used to visualize PECAM-1 (CD31). Alexa 594- and Alexa 633-conjugated secondary antibodies were from Invitrogen (Breda, the Netherlands). Cells were embedded in Vectashield mounting medium (Vector Laboratories, Burlington, CA, USA) and viewed by Confocal Laser Scanning Microscopy using a Zeiss LSM510 (Carl Zeiss, Heidelberg, Germany).

Stimulation of Weibel-Palade body exocytosis

BOECs were plated at 1×10^5 cells/well in collagen-coated 10 cm2 wells. Conditioned medium was refreshed every other day until confluence. The plates were washed twice with serum-free (SF) medium (50% RPMI-1640 and 50% M199-Hepes) supplemented with 1% (v/v) HSA (Cealb, Sanquin, Amsterdam, the Netherlands), 0.3 mg/ml L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. After incubation with SF medium for one hour, the cells were stimulated for one hour with SF medium containing 50 ng/mL phorbol 12-myristate 13-acetate (PMA), 10 μM calcium ionophore A23187, 10 μM epinephrine plus 100 μM IBMX, or 1 U/mL thrombin. The supernatant was collected, centrifuged for 10 min at 10,000 g, supplemented with 10 mM benzamidine and stored at -20°C until use. Cells were subsequently washed and prepared for morphological analysis as described above. FVIII-GFP transduced BOECs were plated at 1×10^5 cells/well in collagen-coated 6 well plates and stimulated with 50 ng/mL PMA as described above.

Inhibition of constitutive release by brefeldin A

Brefeldin A (BFA) treatment of FVIII-GFP transduced BOECs was performed essentially as previously described. Confluent cells were washed twice with SF medium and subsequently incubated for five hours with SF medium supplemented with 5 μM BFA. Medium was collected at several time-points and fresh SF medium with BFA was added. Culture supernatants were centrifuged for 10 min at 10,000 g, supplemented with 10 mM benzamidine and stored at -20°C until further use. FVIII antigen and activity and VWF antigen were determined as described above.

Subcellular fractionation

Subcellular fractionation using Percoll density gradient centrifugation was performed as described with minor modifications. Briefly, BOECs were cultured in two 175 cm² culture flasks until they reached confluence. Cells were washed once with warm HBSS, trypsinized and centrifuged for 10 min at 300 g at 4°C. Cells were suspended in 0.25 M Sucrose, 1 mM EDTA, 20 mM Tris (pH 7.4) and supplemented with 100 μL protease inhibitor cocktail (Sigma-Aldrich, St.-Louis, MO, USA). Cells were homogenized by 20 strokes in a ball-bearing homogenizer (Isobiotec, Heidelberg, Germany) with a 14 micron clearance. The homogenate was centrifuged...
for 10 min at 300g at 4°C. The perinuclear supernatant (PNS) was loaded on a gradient consisting of 40% (v/v) Percoll in 0.42 M Sucrose, 1.68 mM EDTA, 33.7 mM Tris (pH 7.4). The Percoll gradient was centrifuged for 30 min at 100,000 g and 4°C. Fractions of 1 mL were collected from the bottom up and stored at -20°C until further use. FVIII and VWF antigen levels were quantified as described above.

**Statistical analysis**

Student's *t* test was performed with Graphpad Prism version 4.03 (Graphpad Software, San Diego, USA).

**Results**

**Regulated agonist-induced secretion of Weibel-Palade bodies from blood outgrowth endothelial cells**

To study the feasibility of BOECs as a cellular delivery device we isolated BOECs from peripheral blood of healthy individuals. Phenotypic characterization revealed that BOECs displayed typical endothelial 'cobblestone' morphology (Figure 1Ai). Cells were uniformly positive for PECAM-1 (CD31) (Figure 1Aii). Staining for VWF revealed the typical rod-shaped structures representing WPBs (Figure 1Aii). To investigate the possibility of using WPBs as an on-demand protein storage pool, we studied the extent of agonist-induced regulated secretion of VWF from BOECs. Stimulation with various known agonists of WPB exocytosis in HUVEC resulted in release of WPBs and secretion of VWF in the conditioned medium. Stimulation with PMA, calcium ionophore A23187, epinephrine and thrombin resulted respectively in a 6-, 4-, 3.5- and 2-fold increase of the VWF concentration in the conditioned medium compared to the non-stimulated control (Figure 1B). Confocal analysis demonstrated that the non-stimulated BOECs contained numerous WPBs (Figure 1C). In comparison, BOECs stimulated with PMA contained hardly any WPBs (Figure 1C), indicating that the increase in VWF antigen in the conditioned medium resulted from VWF release from the majority of WPBs. We observed clustering of the WPBs in the perinuclear region of the cell after stimulation with epinephrine (Figure 1C; arrow), which is consistent with previous studies on epinephrine-stimulated HUVECs.30

**Lentiviral FVIII-GFP transduction of Blood outgrowth endothelial cells**

BOECs were transduced at passage 5 using a lentiviral vector encoding GFP or human B-domain deleted FVIII-GFP. The transduction did not affect the morphology (Figure 2A) or the growth rate of the transduced
BOECs (Figure 2B), as compared to the non-transduced BOECs. Ten days after transduction, FVIII-GFP-transduced BOECs were approximately 80% positive, as determined by flow cytometry (Figure 2C). This percentage declined slightly to 60% over 31 days of culture (data not shown). Thus, the majority of the cells showed sustained expression of FVIII-GFP.

Quantification of FVIII and von Willebrand factor expression by genetically modified Blood outgrowth endothelial cells

The production levels of FVIII and VWF of genetically modified BOECs were assessed by quantification of VWF and FVIII antigen levels present in conditioned medium. FVIII-GFP-transduced BOECs expressed on average $1.6 \pm 1.0$ pmol/1x10^6 cells/24 h FVIII light chain antigen. To assess the functionality of FVIII secreted by BOECs, we determined the activity/antigen ratio. The activity/antigen ratio of the secreted FVIII was $0.9 \pm 0.2$ after one hour incubation. These data suggest that the FVIII-GFP secreted by BOECs is fully active. This is in agreement with published data from Herder et al. who observed mean activity/antigen ratios of 0.54 to 0.53 after transduction of cord blood derived endothelial progenitor cells (CBECs) with a lentiviral vector encoding FVIII. The activity/antigen ratio declined to 0.2 $\pm 0.1$ after 24 hour incubation. The FVIII-GFP transduced BOECs secreted VWF at $0.45 \pm 0.23$ pmol/10^6 cells/24 h. These levels were similar for non-transduced BOECs (0.49 $\pm 0.35$ pmol/10^6 cells/24 h), indicating that lentiviral transduction and production of high levels of FVIII did not affect VWF synthesis (data not shown).

Co-localization of FVIII and von Willebrand factor in Weibel-Palade bodies

The intracellular localization of FVIII-GFP in transduced BOECs was assessed by confocal microscopy. In the vast majority of the cells, FVIII-GFP co-localized with VWF in WPBs that retained the ability to recruit the trans-membrane protein P-selectin (Figure 3A). As we have described before in HUVEC, storage of FVIII changed the morphology of the WPBs from elongated to round vesicles (Figure 3B inset). As expected, GFP alone did not co-localize with VWF (Figure 3B). Although the shape of the vesicles was altered by the presence of FVIII-GFP, WPBs were still able to recruit the transmembrane protein P-selectin, thereby confirming that FVIII-GFP-containing vesicles correspond to authentic WPBs (Figure 3A).

Exocytosis of Weibel-Palade bodies from FVIII-GFP transduced Blood outgrowth endothelial cells

We subsequently addressed the amount of FVIII-GFP released from transduced BOEC in response to agonists that provoke release of WPBs. Stimulation of FVIII-GFP transduced BOECs with PMA resulted in an 8-fold increase of VWF in the culture medium compared to the non-stimulated control (Figure 4A). In contrast to VWF, there was only a slight 1.3-fold increase of FVIII in the culture medium upon stimulation with PMA (Figure 4B). Our results show that FVIII-GFP is released from WPBs upon stimulation with PMA. However, in quantitative terms, release from this subcellular compartment is limited as opposed to non-stimulated release. It has recently been shown that in absence of a stimulus, so called ‘basal release’ of WPBs accounts for the majority of VWF released by endothelial cells. We investigated whether the rapid accumulation of FVIII-GFP in the conditioned medium of transduced BOECs can also be attributed to basal release of WPBs containing FVIII-GFP. Transduced BOECs were incubated with BFA which prevents constitutive vesicular transport from the trans–Golgi network. Treatment with BFA does not deform pre-formed WPBs and therefore has no effect on basal and regulated secretion. In accordance with data obtained in HUVEC, BFA only partly blocks release of VWF into conditioned medium in BOECs (Figure 5A). In contrast, release of FVIII-GFP is almost completely...
abolished in the presence of BFA (Figure 5B). These findings suggest that, in contrast to VWF, the majority of synthesized FVIII is released in a constitutive manner and not via basal release of WPBs.

**Quantitative determination of the amount of von Willebrand factor and FVIII stored in Weibel-Palade bodies**

These findings indicate that part of the synthesized FVIII-GFP is stored in WPBs whereas the remainder is secreted via the constitutive pathway. To assess this in a quantitative manner, we determined the amount of FVIII-GFP that is stored in WPBs. Homogenates of BOECs were subjected to density gradient centrifugation and the amount of VWF and FVIII was determined in the various fractions by ELISA. A representative fractionation is shown in Figure 6. The first peak (fractions 4-10) corresponds to the high-density WPB-containing fractions, whereas the second peak (fractions 20-25) contains the subcellular fractions derived of organelles of the secretory pathway (endoplasmatic reticulum (ER), Golgi, trans-Golgi network) and constitutively released vesicles. The amount of VWF stored in WPBs was similar for non-transduced (12-15%; Figure 6A) and FVIII-GFP transduced (15-21%; Figure 6B) BOECs. The percentage of FVIII stored in WPBs in FVIII-GFP transduced BOECs was similar to the percentage of VWF stored (15-22%; Figure 6D). The average molar ratio of FVIII to VWF in the WPBs was approximately 1:15. These findings demonstrate that a significant portion of synthesized FVIII is co-targeted to WPBs in transduced BOECs.

**Discussion**

Blood outgrowth endothelial cells provide a cellular delivery device for FVIII that also produces its natural carrier protein VWF. In this study, we analyzed the potential of WPBs to serve as a releasable storage compartment for VWF and FVIII, which may have advantages with respect to high-level, on-demand secretion of
preformed VWF/FVIII complexes that display a prolonged half-life. It has been well-established that the half-life of FVIII is reduced in patients with severe von Willebrand disease. VWF protects FVIII from proteolytic degradation in the circulation and also interferes with the binding of FVIII to its clearance receptors. Recently, VWF has also been shown to inhibit the uptake of FVIII by antigen presenting cells thereby providing a possible modulating effect on the development of an immune response to infused FVIII in patients with hemophilia A. In addition, studies in megakaryocytes have suggested that co-release of VWF and FVIII may protect FVIII from inhibitory antibodies.

We demonstrate that BOECs respond robustly to various stimuli with an increase in the amount of VWF secreted and a reduction in the number of WPBs (Figure 1). We therefore conclude that BOECs do not differ from HUVEC in terms of secretagogue responsiveness and indeed contain a recruitable WPB storage pool. Expression of FVIII-GFP in BOECs resulted in storage of FVIII-GFP in virtually all WPBs (Figure 3A). These vesicles were capable of recruiting the trans-membrane protein P-selectin (Figure 3A), which identifies these FVIII-GFP-containing organelles as true WPBs. FVIII-GFP-containing WPBs within transduced BOECs display round, spherical structures which differ in morphology from the characteristic, elongated structures observed in the absence of FVIII (Figure 3B). Remarkably, in a previous report in which canine FVIII was expressed in canine BOECs, FVIII did not co-localize with VWF in WPBs. This observation suggests that canine FVIII differs from human FVIII in its ability to co-target to WPBs. Alternatively, the amount of canine FVIII stored within WPBs may be too low to allow for detection by indirect staining with polyclonal anti-porcine FVIII antibodies. We have previously observed that the use of intrinsically labeled FVIII-GFP provides a superior means to address the subcellular localization of synthesized FVIII when compared to indirect staining using monoclonal or polyclonal antibodies.

Quantitative assessment of FVIII storage using density gradients revealed that approximately 20% of total intracellular FVIII is present within WPBs (Figure 6D). Nevertheless, the amount of FVIII which can be released upon regulated secretion is limited compared to non-stimulated secretion (Figure 4B). This finding shows that the majority of synthesized FVIII-GFP is released independent of the presence of WPB secretagogues. Non-stimulated secretion may result from release through the

![Figure 4](image-url)
constitutive pathway or from basal secretion of storage organelles that are released in a spontaneous fashion. Here, we show that the majority of FVIII-GFP is released in a constitutive manner and that release of FVIII-GFP does not result from rapid turnover of WPBs in the absence of a stimulus (Figure 5B).

Although the beneficial effect of VWF expression in BOECs in terms of FVIII storage remains to be established, we have demonstrated that lentiviral transduction of BOECs with FVIII-GFP results in high expression levels of FVIII (1.6±1.0 pmol/1×10⁶ cells/24 h) that persist during >30 days of culture. In fact, expression levels are higher than those obtained in established cell-lines. Expression levels were 30-fold higher than those reported in a study in which BOECs were transfectioned using a non-viral transfection method²² and similar to studies in which canine BOECs were transduced using a lentiviral vector encoding canine B-domain deleted FVIII or cord blood-derived endothelial progenitor cells (CBECs) using a lentiviral vector encoding human B-domain deleted FVIII.⁺⁻⁻⁻ Recent studies have shown that endothelial cells are capable of secreting high levels of bio-active, hetero-dimeric FVIII. Therefore, endothelial cells seem to be a particularly suitable delivery device for ex vivo gene therapy for hemophilia A.

It is conceivable that constitutively released FVIII is cleared from the circulation more rapidly compared to the FVIII which is secreted together with VWF via regulated secretion of WPBs. In order to further benefit from co-storage of a VWF/FVIII inducible pool, it may be necessary to enhance the targeting efficiency of FVIII to WPBs. As targeting of FVIII to WPBs is independent of high-affinity interaction between FVIII and VWF,²⁰⁻⁻⁻⁻⁻ we suggest that attempts should be made to increase the amount of VWF stored in WPBs. One approach may be to co-transduce BOECs with VWF. De Meyer et al.¹⁴ have recently demonstrated that WPB formation in VWD type 3 BOECs is restored upon lentiviral transduction with VWF. Another potential approach may be to overexpress the transcription factor KLF2 which has recently been shown to increase the average number of WPBs in HUVEC.⁴⁵

**Figure 6.** Subcellular fractionation and density gradient analysis of FVIII-GFP transduced blood outgrowth endothelial cells. Subcellular fractionation was performed on non-transduced BOECs (A,C) and FVIII-GFP transduced BOECs (B,D). VWF antigen (A,B) and FVIII antigen (C,D) in the various fractions were quantified by ELISA (A,B). The first peak, fractions 4-10, corresponds to the dense WPB fraction, whereas the second peak, fractions 20-25, contains ER, Golgi apparatus and constitutively released vesicles. Shown is a representative experiment of two independent fractionations.

**Authorship and Disclosures**

MB and EAMB performed research, analyzed data and wrote the paper; RPH and NAK contributed essential protocols, techniques and/or materials; JV and KM conceived and designed research, analyzed data and wrote the paper. The authors reported no potential conflicts of interest.

**References**

8. Wilcox DA, Shi Q, Norden P, Habenicht SL, Rosenberg JB,


